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Diastase Alpha-Amylase Immobilization on Gelatin Crosslinked by Glutaraldehyde

Chemistry

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Preeti Lahiri

Chemistry Department, Mahila Maha Vidyalaya, Banaras Hindu University, Varanasi, INDIA

ABSTRACT

α-Amylases are one of the important industrial enzymes that find use in the starch industry for conversion of starch to maltose in preparation of high fructose corn syrup and ethanol. Its immobilization can also improve the enzyme activity and stability in comparison to free enzyme in solution. Various support materials or carrier are used for enzyme immobilization, which may be natural as well as manmade materials. In the present investigation, gelatin has been chosen as an immobilized matrix. α-Amylase was immobilized on gelatin activated by glutaraldehyde as an organic hardener. The conditions of entrapment such as concentration of gelatin, glutaraldehyde and hardening time were optimized for maximum apparent activity. 20 % Gelatin concentration, 10% glutaraldehyde and hardening time of 75 minutes were used and 71.5 % immobilization was achieved. The entrapped α-amylase was characterized in terms of optimum pH, temperature, substrate concentration and compared with those of free enzyme. The optimum pH shifted from 6.5 to 7.5 i.e. towards the basic side upon immobilization. The optimum temperature of gelatin immobilized α-amylase was 65°C as compared to soluble enzyme (55°C) showing an improvement in thermal stability. The Michaelis constant, was estimated for both immobilized and free enzyme. An increase in Michaelis constant, Km from 11.24 to 11.36 mg mL-1 upon immobilization was observed.

Introduction

Immobilization of enzymes is a process having a number of advantages over free enzyme catalysis. This includes increased stability of enzyme, easy recovery of enzyme, easy separation of reactant and product, repeated or continuous use of a single batch of enzyme (1) which will ultimately save the enzyme, labor and overhead costs (2). For many years, immobilized enzymes have been widely used in different industrial processes. There are three basic principles of enzyme immobilization i.e., matrix assisted entrapment of enzyme, adsorption on a solid support, ionic or covalent binding (3-4). Entrapment method is mostly preferred because after immobilization it prevents excessive loss of activity of enzyme, increases stability of enzyme and prevents microbial contamination of enzymes. In entrapment method enzymes are free in solution but because of lattice structure of gel its movement is restricted. Different methods of enzyme immobilization can be used but the most effective method in retaining enzymatic activity is covalent binding in gelatin activated by glutaraldehyde, a chemical organic cross linkers. The immobilized enzyme can contact closely with substrate and remains attached with the inert support matrix and thereby achieving maximum benefits of immobilization.

The method of immobilization and choice of carrier materials should be such that an enzyme faces as little conformational change as possible. Various support materials such as alginate, chitin, chitosan, agarose, agar, gelatine etc. have been used as matrices for enzyme immobilization. Gel formation process is a simple technique and for this, gelatin is preferred as it is relatively in-expensive and non-toxic. Gelatin consists of a mixture of protein and peptides produced by the partial hydrolysis of collagen extracted from the skin, bones and connective tissues of animals such as domesticated cattle, chicken, pig, fish etc. Gelatin is an animal protein unlike many other gelling agents used by the food industry. When gelatin heated it melts into liquid and solidifies when cooled. It forms semisolid gel with water. Gelatin forms a solution of high viscosity in water, which sets to a gel on cooling. There are many reports about immobilization of α-amylase used for the hydrolysis of starch and production of maltose. α-Amylases (α-1,4-glucan-4-glucanohydrolase) found in microorganisms, plants and higher organisms have a wide range of applications in the industrial processes such as food, fermentation, textile, paper, and pharmaceutical industries (5-7). The α-amylase belongs to a family of endo-amylases and it catalyses the initial hydrolysis of starch into shorter oligosaccharides through the cleavage of α-D-(1–4) glycosidic bonds (8-9). The amylase has a three-dimensional structure capable of binding to substrate and it also promote the breakage of the glycosidic links by the action of highly specific catalytic groups.

For enzyme immobilization like amylase, gelatin is used as a matrix activated by glutaraldehyde, a bifunctional agent. Glutaraldehyde is an amine-amine cross-linker frequently used for immobilization of enzymes or hydrophilic coatings on many biomaterials. The hydrophilic coatings especially those containing soluble proteins of the extracellular matrix improve biocompatibility and/or cyto-compatibility of bio-surfaces and afford an opportunity for their application in design of medical implants. Glutaraldehyde has been used in a number of applications where the maintenance of the structural rigidity of the proteins is important. In the present study, alpha amylase (Diastase) has been used for immobilization with gelatin activated by glutaraldehyde. Activity of gelatin immobilized enzyme amylase was charaterized with respect to pH, temperature and substrate concentration. Kinetic parameter was evaluated and compared with free α-amylase.

Experimental

Materials and Methods

Amylase (Diastase), glutaraldehyde, soluble starch and 3,5-dinitrosalicylic acid (DNS), BSA were procured from Himedia Laboratory Pvt. Ltd., (Mumbai). Sodium dihydrogen phosphate, sodium potassium tartrate, sodium hydroxide were purchased from Qualigens (Mumbai). All reagents are A.R grade. DDW was used for preparing solutions.

Enzyme Stock Solution

The diastase alpha amylase was added to 0.1M phosphate buffer (pH 7.0) to the concentration of 1mg/mL. This solution was stored at 4 0C for further experimental work.

Amylase Assay

The activity of α-amylase was estimated by determining the amount of reducing sugars released from starch. The sugars were determined by the method of 3,5-dinitrosalicylicacid (DNS) (10). The starch solution of 1.5% (w/v) was prepared from soluble starch in 0.1M phosphate buffer (pH 7.5). Reaction mixture containing 0.5 ml of the enzyme,

2.5 ml of phosphate buffer (pH 5.5) and 2 ml of 1.5% starch solution was incubated at 40ºC for 15 min. After that 2 ml of DNS was added to interrupt the reaction and the reaction mixture was heated in boiling water for 5 minutes. The -NO2 group of DNSA was reduced to -NH2 group by glucose, which was formed during the enzymatic reaction with starch. After cooling to room temperature, brown-red reduction product was determined spectrophotometrically at λmax 540 nm. In absence of enzyme, a blank experiment was also performed under the identical conditions. The amount of reducing sugar released during the reaction was estimated with the help of maltose standard curve. One unit of enzyme activity (U) was defined as the amount of the enzyme liberating one µmole of reducing sugars as glucose/min under assay condition.

Protein Estimation

The protein content of soluble and immobilized amylase was estimated by the method of Lowry using BSA as a standard (11).

Immobilization of α-Amylase using Gelatin and Glutaraldehyde

In the present work, gelatin solution of different concentrations (10% - 30%) was prepared by dissolving gelatin in distilled water followed by heating at 50°C and thereafter cooling to room temperature. Then 2 ml of enzyme was added to varied concentration of 8 mL gelatin solution with thorough mixing. The suspension was casted on glass plates and when the suspension jellified partially, 10 mL of 10% glutaraldehyde solution was added to each glass plate covered with different concentrations of gelatine. The gel was then left for hardening at 30 0C for 75 minutes. The resulting block of gelatin was then cut into small blocks (4x4 mm) and washed thoroughly with distilled water to remove unbound enzyme and stored in 0.1 M phosphate buffer (pH 7.6) at 4 0C till next use.

Immobilized Enzyme Assay

For assay of immobilized amylase, the appropriate amount of beads of different immobilizations were incubated in 0.1M phosphate buffer (pH 7.5) containing 1.5% starch at 40 0C for 15 minutes. The α-amylase was determined by measuring the reducing sugar released from soluble starch as before. An enzyme unit is defined as the amount of α-amylase that liberates 1 mol of reducing sugar from the substrate per gram of beads under the assay conditions.

Immobilization Efficiency

Immobilization Efficiency (IE) (%) = (I/A-B) X 100

Where, A = Added enzyme (U/g of beads or cubes), B=Unbound enzyme (U/g of beads or cubes), I = Immobilized enzyme (U/g of beads or cubes)

Steady-State Kinetics

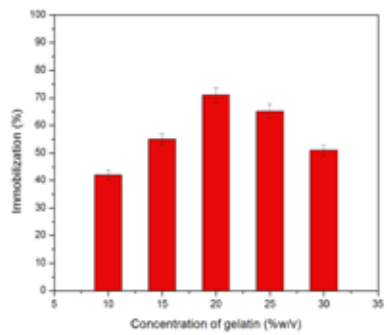
The optimum pH for the immobilized α-amylase was determined by varying the pH of buffers (pH 3-5, sodium citrate buffer; pH 6-8, sodium phosphate buffer; pH 8- 9, NaOH / glycine buffer) from 4.5-9.0. Activity of enzyme was determined by the method described for enzyme assay. The optimal temperature was studied by varying incubation temperature of the reaction mixture from 25-85 °C. Km was determined by Lineweaver - Burk plot with variation in the concentration of starch substrate ranging from 5 to 30 mg mL-1.

Results and Discussion

Immobilization of α-amylase on gelatin

In the present work the enzyme amylase was immobilized on gelatin matrix and covalently cross-linked by the organic hardener glutaraldehyde. The optimization of immobilization of α-amylase in gelatin was done by changing the concentration of gelatin from 5 to 30% (w/v) with glutaraldehyde (10%). It is evident from the Figure 1 that maximum immobilization was obtained at 20% gelatin concentration with 10% glutaraldehyde. It was found from the experimental observations that at low gelatin concentration(less than 5%), unstable and fragile gelatin beads were obtained. This shows poor immobilization due to the larger pore size in the gel and that may be due to leaching of the enzyme. At concentrations above 20%, the percent immobilization was low because of the factor of steric hindrance due to high concentration of gelatin.

Figure 1. Effect of gelatin concentration on percent immobilization



In the absence of glutaraldehyde hardening agent, it was difficult to work out the gelatin matrix (enzyme entrapped within). Further, the glutaraldehyde concentration above 10% may cause the steric hindrance which affects the accessibility of the substrate to amylase. Thus, the optimum immobilization of 71.5% was observed with 20% gelatin and 10% glutaraldehyde concentration within 75 minutes.

Effect of pH on α-Amylase (soluble)

Soluble enzyme can have altered pH optima upon immobilization. The effect of pH on the activity of free and immobilized amylase was shown in Figure 2. The pH optima of soluble and immobilized amylase were 6.5 and 7.5 respectively, i.e., a shift of 1unit towards the basic side was resulted from immobilization of α-amylase on gelatin. A similar trend of shift in pH towards the basic side (from 5.6 to7.0) was reported (12) from immobilization of a-amylase on both chitosan and amberlite. In addition, based upon the surface and residual charges on the solid matrix and the nature of the enzyme bound, the pH value in the nearby vicinity of the enzyme molecule may change that brings a shift in the pH of the enzyme activity.

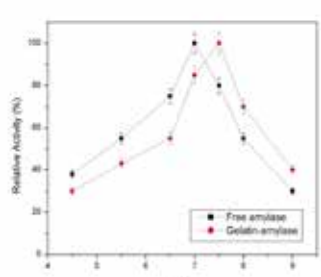


Figure 2. Effect of pH on soluble and gelatin immobilized α-amylase activity

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Effect of Temperature

The effect of temperature on the activity of free and immobilized amylase was shown in Figure 3. The soluble enzyme showed a temperature optimum of 55°C and this value shifted to 65°C for the immobilized enzyme. This increase in the temperature optima may be due to the conformational integrity of the enzyme structure upon covalent binding to the support material (13-14). Also, higher temperature optima in enzymes are attractive because rate of reactions is faster and there is less microbial contamination of food materials.

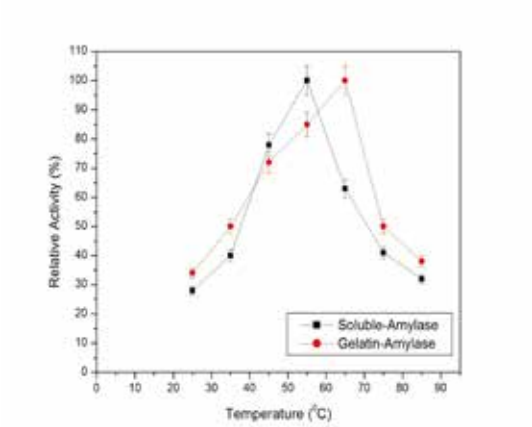

Figure 3. Effect of temperature on soluble and gelatin immobilized α-amylase

Kinetic Analysis

Kinetic parameters of both free and immobilized α-amylase were measured. For both forms of α-amylase, Michaelis- Menten type kinetic behavior was observed. The Km and Vmax values as determined from the Graph Pad Prism Software were found to be 11.24 mg/ml and 1.52 µmole/min and 11.36 mg/ml and 1.38 µmole/min for free and immobilized enzyme (data not shown) respectively . Therefore, for immobilized form of α-amylase, Vmax is decreased and Km is slightly increased as compared to free form. The Km value is considered as the affinity of the enzymes to substrates and the lower value of Km emphasizes the higher affinity between enzymes and substrate (15). The present results show that affinity of enzyme to its substrate was slightly decreased upon immobilization. This could be due to the leaching of enzyme to some extent.

Conclusions

In the present work, it has been investigated that the factors such as concentrations of gelatin, glutaraldehyde and hardening time had significant effects on immobilization yield in gelatin-amylase beads. Temperature and pH optima of α-amylase in beads were shifted; this could increase the applicability of the enzyme. It was found from the Km values that after immobilization there is a small decrease in substrate affinity of α-amylase. The enzyme immobilization in gelatin is simple, cost effective and safe. Since the gelatin is naturally occurring, nontoxic and biodegradable, the results of the present investigation are useful in the development of a general approach for carbohydrate hydrolysis in food/feed industry.

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